

# Hedgehog Is an Anti-Inflammatory Epithelial Signal for the Intestinal Lamina Propria

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**BACKGROUND & AIMS:** Epithelial Hedgehog (Hh) ligands regulate several aspects of fetal intestinal organogenesis, and emerging data implicate the Hh pathway in inflammatory signaling in the adult colon. Here, we investigated the effects of chronic Hh inhibition *in vivo* and profiled molecular pathways acutely modulated by Hh signaling in the intestinal mesenchyme. **METHODS:** The progression of inflammatory disease was characterized in a bi-transgenic mouse model of chronic Hh inhibition (VFHhip). In parallel, microarray and bioinformatic analyses (Gene Ontology terms overrepresentation analysis, hierarchical clustering, and MeSH term filtration) were performed on isolated cultured intestinal mesenchyme acutely exposed to Hh ligand. **RESULTS:** Six- to 10-month-old VFHhip animals exhibited villus smooth muscle loss and subsequent villus atrophy. Areas of villus loss became complicated by spontaneous inflammation and VFHhip animals succumbed to wasting and death. Phenotypic similarities were noted between the VFHhip phenotype and human inflammatory disorders, especially human celiac disease. Microarray analysis revealed that inflammatory pathways were acutely activated in intestinal mesenchyme cultured in the absence of epithelium, and the addition of Hh ligand alone was sufficient to largely reverse this inflammatory response within 24 hours. **CONCLUSIONS:** **Hh ligand is a previously unrecognized anti-inflammatory epithelial modulator of the mesenchymal inflammatory milieu. Acute modulation of Hh signals results in changes in inflammatory pathways in intestinal mesenchyme, while chronic inhibition of Hh signaling in adult animals leads to spontaneous intestinal inflammation and death. Regulation of epithelial Hh signaling may be an important mechanism to modulate tolerogenic versus proinflammatory signaling in the small intestine.**

**Keywords:** Hedgehog Signaling; Inflammatory Bowel Disease; Celiac Disease; Myeloid Cells.

**F**unctional intestinal immunity requires a precise balance of proinflammatory and tolerogenic influences to both protect against infectious disease and prevent aberrant inflammation. When inappropriate proinflam-

matory signals dominate, as is the case in inflammatory bowel disease (IBD) and celiac disease, significant tissue damage results, leading to substantial patient morbidity. Although IBD is thought to result from aberrant chronic activation of the immune system in response to luminal flora,<sup>1</sup> celiac disease involves an immune response to dietary gluten. In both cases, the disease etiology involves a complex interplay between luminal agents, the intestinal epithelium, and the immune elements within the underlying mesenchymal compartment. Indeed, through secretion of molecules such as thymic stromal lymphopoietin (TSLP), the epithelium is a critical modulator of the mesenchymal inflammatory response.<sup>2–4</sup> However, other epithelial modulators of inflammation must exist, since dendritic cells isolated from mice null for the TSLP receptor do not show defects in their ability to activate regulatory T cells<sup>5</sup> and TSLP is not capable of completely replacing epithelial cells as an anti-inflammatory modulator for intestinal myeloid populations.<sup>3</sup>

A second epithelial signaling molecule recently linked to IBD is Hedgehog.<sup>6</sup> Both Indian (Ihh) and Sonic (Shh) Hedgehog are secreted by the intestinal epithelium and signal in a paracrine manner to the mesenchyme.<sup>7</sup> Ihh and Shh are involved in a number of developmental patterning events in the intestine<sup>8–10</sup> and are also expressed in the adult gastrointestinal tract.<sup>7</sup> Among the target cells that respond to Hh signals in the adult colon are dendritic cells and macrophages.<sup>6</sup> Interestingly, a nonsynonymous single nucleotide polymorphism in GLI1, a downstream transcription factor and direct target of Hh signaling,<sup>11</sup> is associated with IBD in 3 large European populations; the variant GLI1 protein shows reduced transactivation activity in transfected cells.<sup>6</sup> In accordance with the idea that reduced Hh signals might predispose to inflammation, Gli1<sup>+/−</sup> mice exhibit increased susceptibility to dextran sodium sulfate with

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*Abbreviations used in this paper:* Hh, Hedgehog; IBD, inflammatory bowel disease; Ig, immunoglobulin; Ihh, Indian Hedgehog; IL, interleukin; Shh, Sonic Hedgehog; TSLP, thymic stromal lymphopoietin; VFHhip, 12.4Kvii-Cre x 12.4Kvii-lacZ-flox-Hhip.

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markedly up-regulated interleukin (IL)-23/IL-17 signaling.<sup>6</sup> These data suggest the novel hypothesis that the adult intestinal epithelium might use Hh ligands to modulate the tolerogenic response of the lamina propria, but the potential genetic targets of Hh signaling involved in this regulation have not been identified.

The objectives of the current investigation were 2-fold: (1) to understand how a chronic reduction in Hh signals would impact intestinal health and (2) to learn more about the target genes and pathways that are altered by acute changes in Hh signaling in the intestine. To accomplish the first goal, we carried out a long-term analysis of a bi-transgenic mouse model of Hhip (Hh-interacting protein) overexpression, 12.4KVil-Cre x 12.4KVil-flox-LacZ-flox-Hhip (which we call VFHhip). This model, which is described in detail elsewhere (Zacharias et al, submitted manuscript), allows analysis of the phenotypes arising from chronic reduction in Hh levels, as Hhip is a pan-Hh inhibitor<sup>12</sup> and VFHhip animals show clear down-regulation of the Hh pathway by 1 month of life. Early phenotypic changes in the VFHhip model include crypt expansion followed by reduction in villus smooth muscle (Zacharias et al, submitted manuscript). Here, we show that older VFHhip animals exhibit villus atrophy and develop spontaneous small intestinal inflammation.

To learn more about the inflammatory pathways acutely regulated by epithelial Hh proteins, we performed microarray analyses on small intestinal mesenchyme samples (from wild-type mice) to which purified Hh ligands were added. The results of this microarray indicate that intestinal Hh signaling is primarily pro-myogenic and anti-inflammatory. Further, we show that when intestinal mesenchyme is cultured for 48 hours without epithelium (the Hh source), pro-inflammatory genes are up-regulated. Readdition of Hh ligand alone results in robust down-regulation of the same genes. Thus, we propose that epithelial Hh ligands are important homeostatic modulators of small intestinal tolerance and suggest that chronically reduced Hh signaling may promote intestinal inflammatory disease.

## Materials and Methods

### Microarray and Bioinformatic Analysis

E18.5 intestinal mesenchyme was isolated, cultured, and treated with Hh ligand as described previously.<sup>8</sup> RNA preparation, microarray analysis, and bioinformatics processing were performed as described in Supplementary Materials and Methods. Comparisons used to identify differentially expressed genes are shown schematically in Supplementary Figure 1. Array data are available in the GEO database (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE17840>).

### Analysis of VFHhip Animals

The generation of VFHhip animals (12.4KVil-flox-LacZ-flox-Hhip x 12.4KVil-Cre bi-transgenic mice) is de-

scribed elsewhere (Zacharias et al, submitted manuscript). Briefly, a 12.4-kb genomic fragment containing the upstream region of the mouse villin gene<sup>13</sup> was linked to a truncated version of the Hhip complementary DNA, which lacks the transmembrane region.<sup>8</sup> A floxed LacZ cassette was placed between the villin regulatory sequence and the Hhip complementary DNA; Cre activity (driven by 12.4KVil-Cre<sup>13</sup>) results in the loss of the LacZ cassette, activation of the Hhip complementary DNA, and intestine-specific inhibition of Hh signaling. Tissue processing, immunostaining, and functional analysis of isolated myeloid cells were performed as described in Supplementary Materials and Methods.

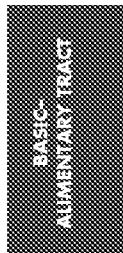
## Results

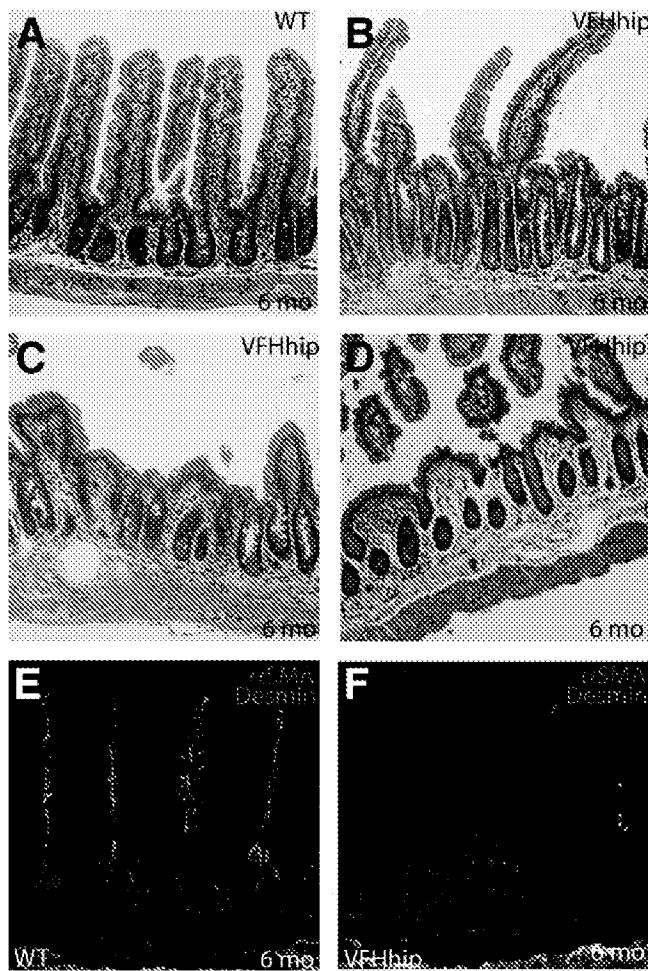
### *Chronic Inhibition of Adult Intestinal Hh Signals Leads to Villus Loss and Spontaneous Inflammation*

Because recent data suggest that a 50% reduction in Hh signals leads to inflammation of the adult colon,<sup>6</sup> we wished to examine the long-term effects of moderate Hh inhibition in the adult intestine. In VFHhip bi-transgenic mice, Cre expression leads to activation of the pan-Hh inhibitor, Hhip. Hhip expression begins perinatally, several days later than in the previously described 12.4KVil-Hhip transgenic founders.<sup>8</sup> Quantitative polymerase chain reaction evaluation of Hh pathway activity in these mice shows that by 1 month of age, *Gli1* expression is reduced to approximately 30% of normal expression, indicating significant pathway inhibition (Zacharias et al, submitted manuscript). During the first 3 months of life, VFHhip mice exhibit expansion of the proliferative crypt region of the epithelium, mislocalization of intestinal subepithelial myofibroblasts, and progressive loss of villus smooth muscle (Zacharias et al, submitted manuscript).

Here, we further aged VFHhip animals and examined them for intestinal alterations during a period of 6 to 10 months. At 6 months of age, all VFHhip animals exhibited deep crypts and areas of patchy villus loss (Figure 1A-D). Regions devoid of villi were often complicated by inflammation in the lamina propria (Figure 1C and D). Villus smooth muscle was dramatically reduced, as shown by loss of cells positive for both  $\alpha$ -smooth muscle actin and desmin (Figure 1E and F). However, there was no evidence of weight loss or wasting at this time.

Between 6 and 10 months of age, areas of villus loss were interspersed with regions exhibiting villus blunting, crypt hyperplasia, mucous cell expansion, and lamina propria inflammation (Figure 2A-D). These lesions bore resemblance to those found in human celiac disease; however, no increase in intraepithelial lymphocytes was seen in inflamed areas. Inflammation was prominent in the lamina propria and with age, was seen more frequently in submucosal regions (Figure 2D). Lesions of variable size and severity were identified throughout the





**Figure 1.** Chronic inhibition of Hedgehog (Hh) signaling leads to villus atrophy. Studies were done in 12.4KvII-Cre  $\times$  12.4KvII-flox-LacZ-flox-Hhip (VFHhip) double-transgenic mice and their single transgenic (ST) littermates (carrying either 12.4KvII-Cre or 12.4KvII-flox-LacZ-flox-Hhip transgene). (A) Villus structure is normal in 6-month-old ST mice. (B) Six-month-old VFHhip animals exhibit villus loss with dramatic crypt expansion. (C, D) Regions of villus loss and inflammation in 6-month-old VFHhip mice. (E, F)  $\alpha$ -smooth muscle actin/desmin double staining for visualization of myoblasts (green), myofibroblasts (red), and differentiated smooth muscle (yellow). VFHhip animals exhibit extensive loss of villus smooth muscle by 6 months of age.

length of the small intestine. The extent, severity, and timing of inflammation was somewhat variable but appeared to be progressive; a time course of disease progression is provided in Supplementary Figure 2A. The colon in these mice exhibited little phenotype, likely because the villin promoter, which drives expression of the Hhip transgene, is much stronger in the small intestine than in the colon.<sup>13</sup> The level of Hhip in the colon of these mice may not be sufficient to inhibit Hh signals.

Interestingly, VFHhip animals developed dermatitis more frequently than single transgenic littermates. By 10 months of age (or at the time of death), 8 of 10 VFHhip animals had developed visible dermatitis, whereas only 4 of 15 co-housed single transgenic littermates showed dermatitis (Supplementary Figure 2A). Because immuno-

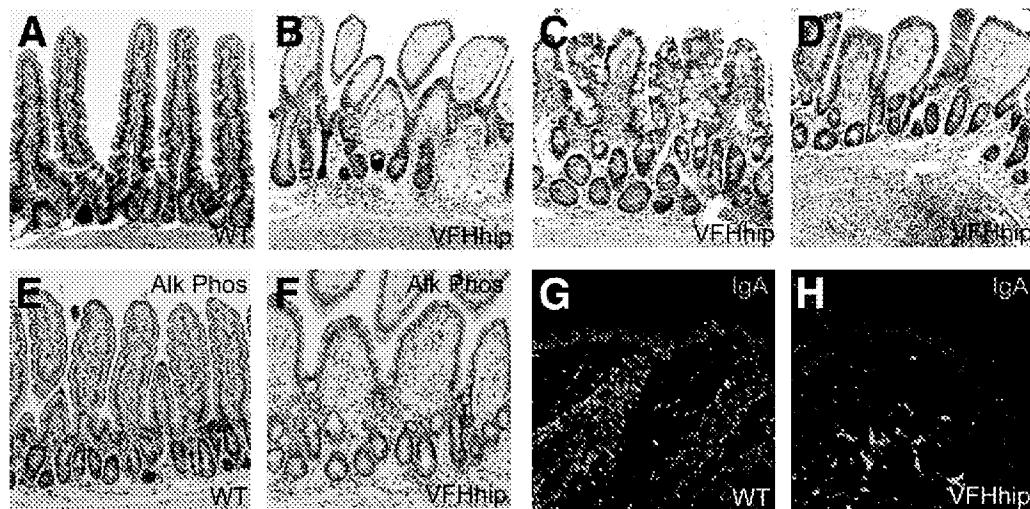
globulin (Ig) A deposition is a complication of a characteristic dermatitis in human celiac disease,<sup>14</sup> we examined the skin lesions in 5 VFHhip animals and 5 single transgenic littermates for IgA deposits. IgA was observed in the inflamed skin of 3 of 5 VFHhip animals but not in any of the single transgenic littermates (Figure 2E and F).

Older VFHhip animals stochastically developed diarrhea and rapid weight loss, culminating in death. By 10 months of age, the entire cohort of bi-transgenic VFHhip animals ( $n = 10$ ) had died (Supplementary Figure 2B), while all single transgenic littermates housed with VFHhip animals remained alive ( $n = 14$ ). Because animals with these late-stage changes also showed signs of malabsorption (eg, weight loss, diarrhea, lethargy), we assessed the differentiation status of the remaining epithelium. Immunostaining with alkaline phosphatase, a marker of differentiated epithelial cells, revealed a striking lack of differentiated enterocytes (Figure 2G and H), providing potential insight into the clinical wasting displayed by VFHhip animals. Together, these data indicate that long-term inhibition of intestinal Hh signaling is associated with crypt hyperproliferation, progressive villus loss, and severe inflammatory disease with phenotypic similarities to human inflammatory conditions.

### Hh Treatment Inhibits Expression of Inflammatory Genes in Isolated Mesenchyme

To address whether the development of inflammatory disease in older VFHhip mice is due to direct modulation of inflammatory pathways by Hh signals or whether progressive tissue changes (eg, barrier breaks during villus atrophy) predisposed to inflammatory disease, we examined the acute transcriptional response of intestinal mesenchyme to the addition of Hh ligand. It is known that the epithelium is the primary source of Hh signals throughout fetal and adult life and these Hh signals impact paracrine targets in the mesenchyme, including myofibroblasts, smooth muscle precursors, and differentiated smooth muscle cells.<sup>7</sup> We previously showed that whole intestinal mesenchyme from E18.5 mice can be cleanly isolated from the epithelium.<sup>8</sup> Culture of this isolated mesenchymal fraction for 48 hours in the absence of epithelium results in loss of Hh signal transduction as measured by expression of the direct target gene and Hh transcription factor *Gli1*; Hh signal transduction can be robustly reactivated within the mesenchyme 24 hours after addition of recombinant Shh or Ihh ligand to the mesenchymal cultures (Zacharias et al, submitted manuscript). Thus, isolated cultured mesenchyme is an appropriate model to identify genes and pathways that are acutely modulated by Hh signaling.

Isolated intestinal mesenchyme was cultured for 48 hours without passage, and then exposed to recombinant Shh or Ihh ligand (or vehicle) for 24 hours. We identified probe set expression values that were modulated at least 1.5 fold (and  $P \leq .05$ ) by Hh treatment. Unexpectedly, we



**Figure 2.** 12.4KvII-Cre x 12.4KvII-flox-LacZ-flox-Hhip (VFHhip) animals develop spontaneous small intestinal inflammation. (A–D) H&E staining of single transgenic (ST) (A) and VFHhip (B–D) mice at 8–10 months of age. (B) VFHhip animals exhibit inflammatory cells in the lamina propria beneath blunted villi. (C) Some focal areas of villus blunting, crypt expansion, and goblet cell hyperplasia resemble the histology of human celiac disease. (D) In focal areas, inflammation extends submucosally. (E, F) Staining for immunoglobulin (Ig) A in skin lesions of ST and VFHhip mice. VFHhip mice frequently show dermatitis (Supplementary Figure 2B) and 60% of these lesions contain depositions of IgA (F), while skin lesions observed in ST animals (E) do not. (G, H) In ST, alkaline phosphatase staining is prominent on villus tips (G), while blunted villi in VFHhip animals exhibit loss of functional absorptive surface (H).

found more down-regulated than up-regulated probe sets with both Shh and Ihh treatment (Supplementary Tables 1 and 2). There was considerable overlap in probe sets regulated by Shh and Ihh: 27 common up-regulated probe sets, including the known targets, *Ptc1* and *Gli1*, and 75 common down-regulated probe sets (Supplementary Tables 3 and 4), further validating the genes associated with these probe sets as likely downstream targets (either direct or indirect) of Hh signaling.

We carefully examined the array data for evidence of a differential response to Shh versus Ihh. Twenty-five potential differential targets with a fold difference of  $>1.2$  and  $P \leq .05$  were identified; we utilized a low cutoff of 1.2-fold in order to stringently assess differentially regulated targets. However, each of these targets was regulated with both Shh and Ihh treatment in the same direction, although with subtle expression differences (data not shown); these differences may therefore be a result of recombinant ligand activity in vitro rather than true differential activation by Shh and Ihh ligand. Thus, we found no solid evidence for a differential response of intestinal mesenchyme to Shh versus Ihh in these studies.

We proceeded to examine those targets that were significantly regulated ( $\geq 1.5$  fold) by both Shh and Ihh in cultured mesenchyme (Supplementary Tables 3 and 4). First, we compared the frequency of most highly represented Gene Ontology Biological Process terms in the group of probe sets up-regulated by both Hh ligands with the frequency of these same terms in the list of probe sets expressed in whole mesenchyme (Figure 3A). Second, we utilized DAVID Functional Annotation to identify major pathways modulated in Hh-treated mesenchyme (Table

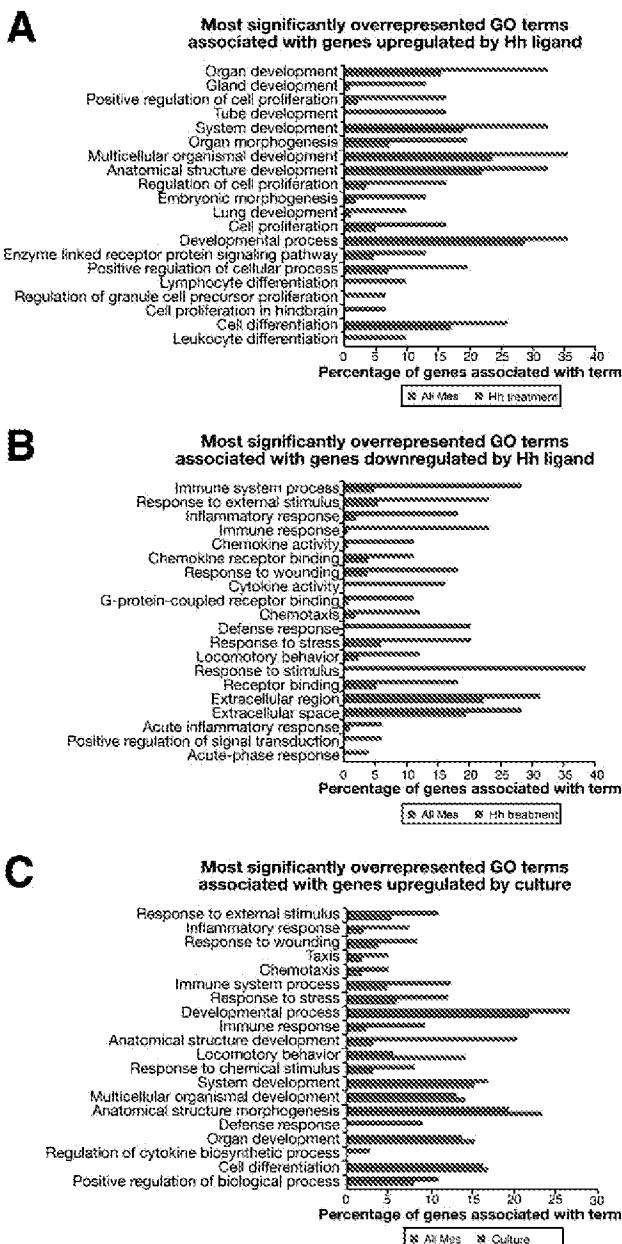
1). Both analyses revealed that genes up-regulated by Hh are strongly associated with developmental processes. MeSH anatomy filtration was used to examine the cell-type specificity of up-regulated probe sets. Six of the 10 top cell types linked to these probe sets were muscle-related (Supplementary Table 5), consistent with previous studies that show roles for Hh in intestinal smooth muscle development.<sup>8–10</sup>

Analysis of probe sets down-regulated by both Shh and Ihh revealed a striking result: these probe sets were strongly associated with genes that exhibit pro-inflammatory function (Figure 3B). Major intestinal inflammatory players, such as *Il-1 $\beta$* , *Il-6*, and several key CC and CXC class chemokines, are down-regulated by Hh signaling (Supplementary Table 4). In addition, several genes important in the maturation and function of myeloid immune lineages, including *CD11b/Itgam* and *CD14*, are down-regulated in response to Hh signals. DAVID functional annotation of these genes suggests that Hh signaling modulates genes associated with immune system process and stimulus response (Table 1). Indeed, MeSH anatomy filtration reveals that the majority of genes regulated by Hh are expressed in myeloid and other immune cell types, bolstering the conclusion that the activation of Hh signaling in intestinal mesenchyme regulates inflammatory processes in these cells (Supplementary Table 6).

#### Culture of Intestinal Mesenchyme Results in Activation of Inflammatory Pathways

In order to better contextualize the gene expression changes in isolated mesenchyme induced by Hh





**Figure 3.** Indian Hedgehog (Ihh) and Sonic Hedgehog (Shh) regulate developmental and inflammatory pathways in isolated intestinal mesenchyme. Overrepresented Biological Processes were identified using DAVID for (A) genes up-regulated by Hh, (B) genes down-regulated by Hh, and (C) genes up-regulated by transition to culture. The percentage of genes associated with each of the 20 most overrepresented processes (identified by *P* value, most significant at the top, blue bars) were compared to the percentage of genes associated with the same process in the list of all mesenchymally enriched genes (citation 26, red bar).

ligands, we sought to profile the changes associated with the transition of mesenchyme to culture. We compared gene expression in freshly isolated E18.5 mesenchyme<sup>15</sup> to cultured mesenchyme treated with vehicle (the control set for the Hh treatment analysis here); this vehicle-treated mesenchyme had been in culture for a total of 72

hours at the time of analysis. Over 7000 probe sets showed changes >2-fold during transition to culture. The vast majority of these genes (~5500) exhibited changes between 2- and 3-fold; we therefore chose a cutoff of  $\geq 5$  fold to identify those regulated processes that are mostly strongly modified by the transition to culture. This comparison identified 358 up-regulated probe sets and 76 down-regulated probe sets (Supplementary Table 7) with expression changes  $>5$ -fold in cultured versus uncultured mesenchyme.

Importantly, *Gli1* (−7.3-fold), *Ptch1* (−4.3-fold), and *Hhip* (−12.5-fold), all direct early target genes of Hh signaling,<sup>11,12,16</sup> were each robustly down-regulated in cultured mesenchyme, providing further evidence that removal of epithelium reduces the activity of the Hh pathway (Supplementary Table 7). DAVID Functional Annotation analysis revealed that the major up-regulated gene categories during entry to culture include inflammatory response, cytokine signaling, and positive regulation of growth and proliferation (Figure 3C and Supplementary Table 8). Major down-regulated processes include muscle and extracellular matrix related-pathways (Supplementary Table 9).

### ***Hh Ligands Are Sufficient to Blunt the Inflammatory Response Present in Intestinal Mesenchyme Following Removal of the Intestinal Epithelium and Entry to Culture***

Removal of the epithelium and culture of the mesenchyme is likely to modulate more than just Hh signaling in the mesenchyme, since the epithelium is the source of a number of additional signals that can modify mesenchymal cell response.<sup>15</sup> We therefore examined the extent to which activating Hh signaling alone could alter the direction of pathway regulation (ie, can Hh ligand cause a gene that was down-regulated during transition to culture to instead be up-regulated?).

First, we identified those probe sets that were both down-regulated by transition to culture (and removal of the source of Hh ligand) and up-regulated by the addition of both Shh and Ihh. This cohort of genes (Supplementary Table 10) includes the known direct Hh targets *Ptch1* and *Gli1*, as well as a recently identified direct target *MyoD* (Zacharias et al, submitted manuscript). These findings suggest that this group of genes is likely to contain other novel direct Hh targets. Functional analysis with DAVID indicates that the major process related to this group of probe sets is organ development (Supplementary Table 11).

Next, we identified those probe sets that were both up-regulated by transition to culture and down-regulated by the addition of Hh ligand (Supplementary Table 12). Hierarchical clustering identified two clusters of probe sets that are expressed at low levels in freshly isolated mesenchyme, are up-regulated during the transition to culture, and are down-regulated by addition of Hh ligand

**Table 1.** DAVID Functional Annotation Clusters for Genes Regulated by Shh and Ihh

Functional cluster	Enrichment score	Top term	Top term		
			FE	P value	FDR
Processes associated with genes up-regulated by Shh and Ihh treatment of intestinal mesenchyme					
1	2.78	GO:0035295	Tube development	15.52	2.28E-04
2	1.6	INTERPRO: IPR013783	Immunoglobulin-like fold	6.29	1.92E-03
3	1.24	GO:0030098	Cytokine activity	17.5	1.16E-02
4	1.19	UP_SEQ_FEATURE	Disulfide bond	2.37	1.80E-02
5	1.17	GO:0048513	Carbohydrate binding	4.41	1.41E-04
6	1.13	SP_PIR_KEYWORD	Transmembrane protein	7.57	1.34E-02
7	0.89	GO:0044249	Cellular biosynthetic process	3.17	6.06E-02
8	0.78	GO:0048598	Embryonic morphogenesis	12.8	3.18E-03
9	0.75	GO:0044238	Primary metabolic process	1.39	7.52E-02
10	0.64	GO:0006955	Immune response	4.51	5.15E-02
Processes associated with genes down-regulated by Shh and Ihh treatment of intestinal mesenchyme					
1	12.22	GO:0009605	Response to external stimulus	10.52	5.31E-17
2	10.81	GO:0002376	Immune system process	7.4	3.36E-17
3	8.98	GO:0005125	Cytokine activity	15.54	6.26E-14
4	3.54	GO:0042221	Response to chemical stimulus	7.15	3.20E-09
5	2.3	GO:0030246	Carbohydrate binding	5.96	3.50E-04
6	1.78	GO:0007159	Leukocyte adhesion	61.89	9.95E-04
7	1.74	GO:0008201	Heparin binding	10.86	3.04E-02
8	1.67	GO:0009967	Positive regulation of signal transduction	12.84	9.52E-05
9	1.54	KEGG_PATHWAY	Toll-like receptor signaling pathway	6.67	1.58E-03
10	1.44	SP_PIR_KEYWORD	Innate immunity	13.44	2.05E-02

FDR, false discovery rate; FE, fold enrichment.

(Figure 4A). These probe sets are associated with genes that are clearly pro-inflammatory (Table 2 and Figure 4B); several CC and CXC cytokines are strongly up-regulated by transition to culture and also markedly down-regulated by addition of Hh ligand. Additional genes on this list include *IL-1b*, *IL-6*, TLR2, and cell surface markers expressed on myeloid cells including *CD14* and *CD11b*. These data strongly suggest that (1) epithelial Hh acts as an anti-inflammatory signal, (2) removal of the epithelium has a pro-inflammatory effect on the mesenchyme, and (3) addition of Hh ligand alone can largely substitute for the anti-inflammatory activity provided by the epithelium.

#### ***Hh Signals Act Directly on CD11b<sup>+</sup> Myeloid Cells to Blunt the Release of IL-6***

The MeSH data indicate a clear association of Hh down-regulated genes with expression in myeloid cell

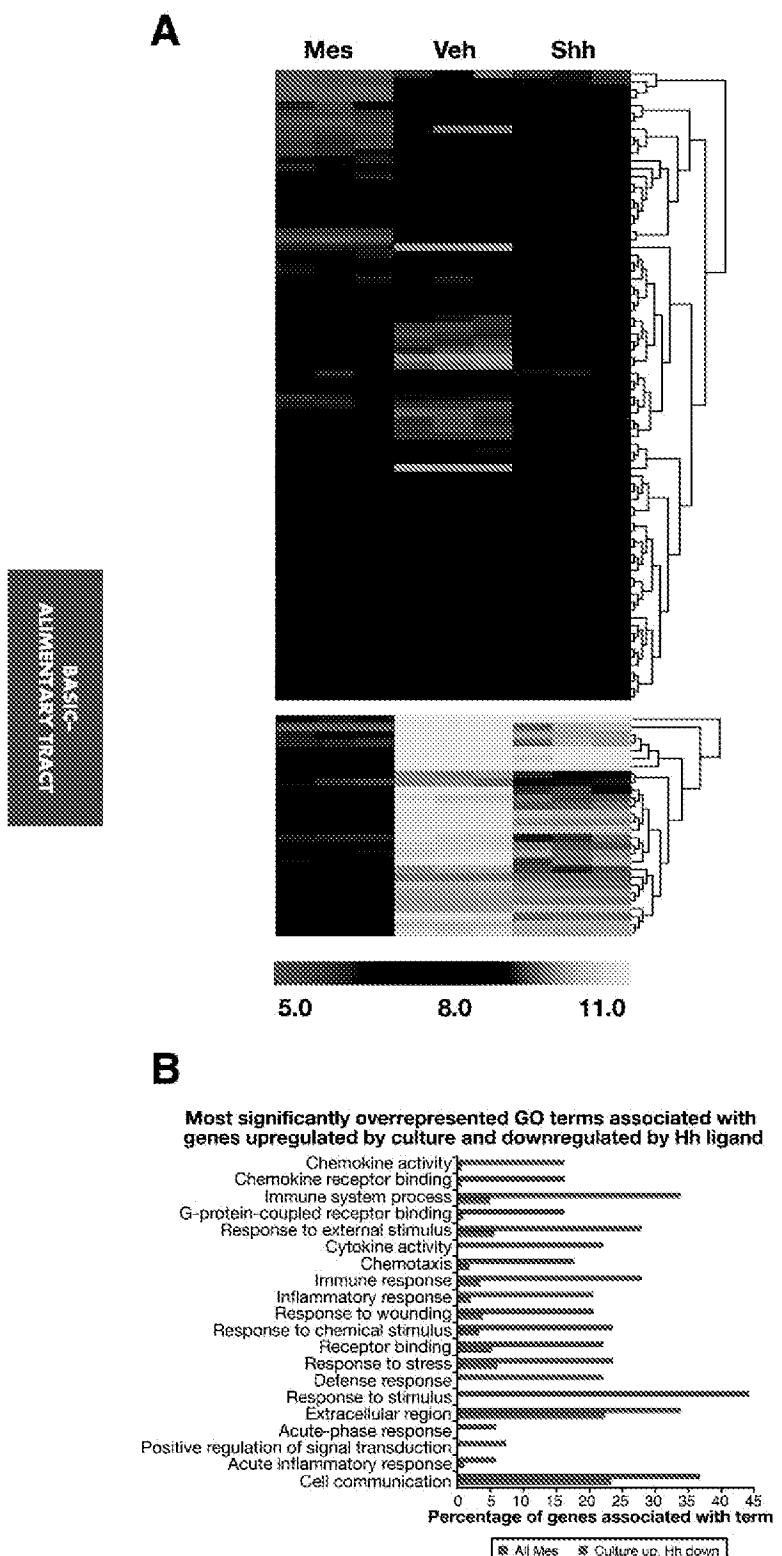
lineages (Supplementary Table 6). Myeloid cells of other organs, including the colon,<sup>6</sup> respond directly to Hh signals, but small intestinal myeloid lineages have unique characteristics,<sup>3,17,18</sup> and we wished to assess whether these cells also responded to Hh signals. To this end, we utilized Gli1<sup>+/LacZ</sup> animals, which express β-galactosidase under the control of the Gli1 locus. Because Gli1 is a direct Hh target expressed only in cells that respond to Hh signaling,<sup>19</sup> β-galactosidase expression is a sensitive readout of Hh response in these animals. We examined β-galactosidase expression in small intestinal CD3<sup>+</sup> T cells, CD19<sup>+</sup> B cells, and in CD11b<sup>+</sup> and CD11c<sup>+</sup> myeloid cells. To carefully identify only those cells that expressed both β-galactosidase and immune markers, we used confocal microscopy to evaluate serial 0.3-μm optical sections. While CD3<sup>+</sup> T cells and CD19<sup>+</sup> B cells do not appear to respond to Hh signals under homeostatic conditions (Figure 5A and B), some, but not all, CD11b<sup>+</sup>

**Table 2.** DAVID Functional Annotation Clusters for Genes Up-regulated by Culture and Down-Regulated by Hedgehog Ligand

Functional cluster	Enrichment score	Top term	Top term		
			FE	P value	FDR
1	8.89	GO:0008009	Chemokine activity	79.64	6.62E-17
2	1.88	KEGG_PATHWAY	Toll-like receptor signaling pathway	8.89	3.84E-04
3	1.88	SP_PIR_KEYWORD	Immune response	9.69	1.64E-03
4	1.62	GO:0043123	Positive regulation of IKK $\beta$ kinase/NF-κB cascade	21.60	8.16E-03
5	1.53	GO:0004871	Signal transducer activity	1.75	1.08E-02
6	0.98	GO:0009310	Amine catabolic process	14.16	1.83E-02

FDR, false discovery rate; FE, fold enrichment; NF, nuclear factor.

and CD11c<sup>+</sup> myeloid cells respond directly to Hh signals (Figure 5C and D). These results were further confirmed using intestinal mesenchyme isolated from Gli1<sup>LacZ/+</sup> mice (Figure 5E and F). When these cells were stained with antibodies to CD11b, all cells with a dendritic-like morphology were strongly CD11b<sup>+</sup> and LacZ<sup>+</sup> (*white arrow*



rows, Figure 5E and F). In contrast, CD11b<sup>+</sup> cells with a larger, more spread morphology were only weakly positive for LacZ (*dotted circle*, Figure 5E and F).

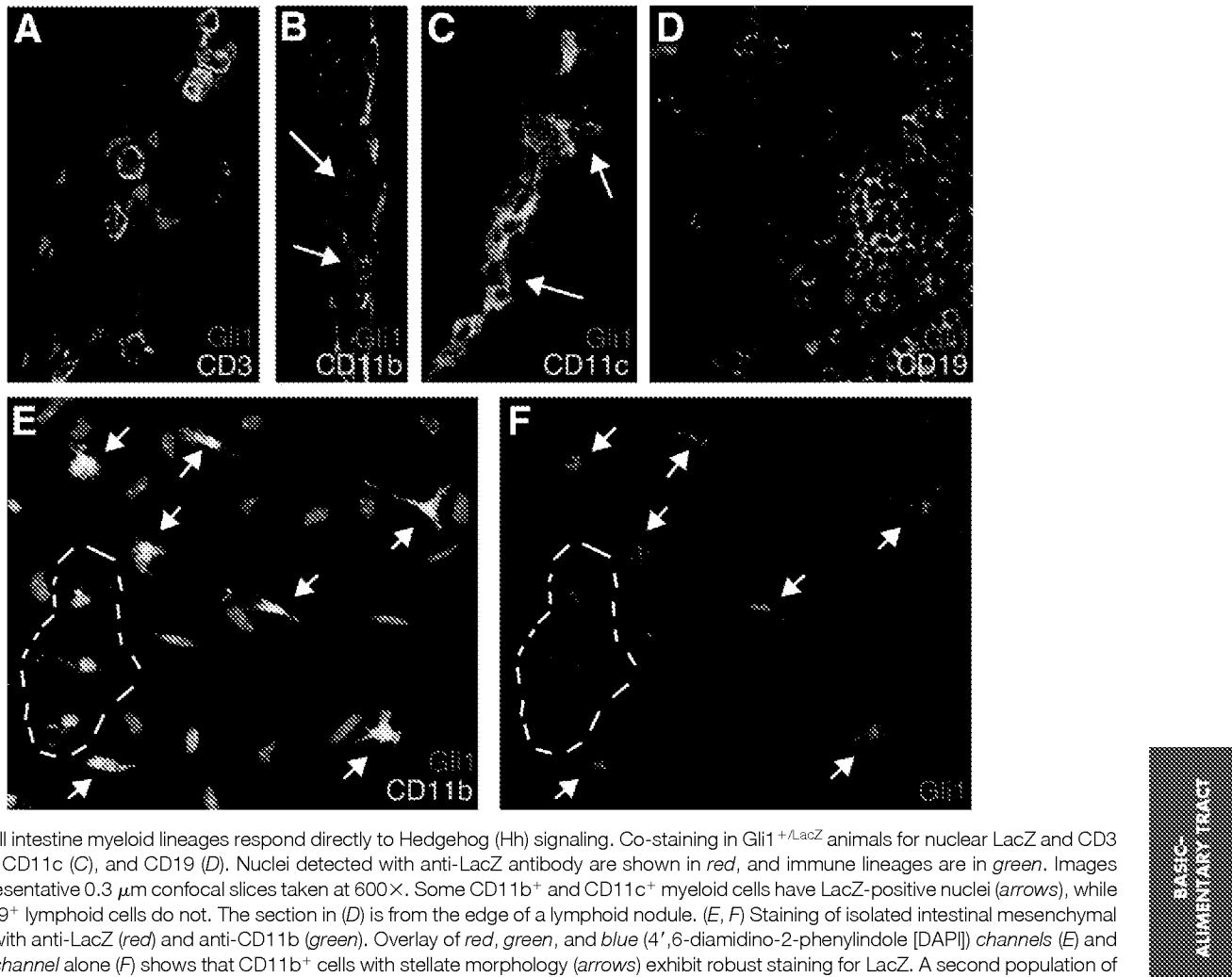
Finally, to examine the functional effect of Hh ligands on CD11b<sup>+</sup> cells, we isolated these cells from small intestinal lamina propria, exposed them to Ihh or Shh in culture, and measured the release of the proinflammatory cytokine, IL-6. Treatment with both Hh ligands severely blunted the release of IL-6 (Figure 6). Myeloid lineages are known to be immunomodulatory in the small intestine<sup>3</sup> and these findings, in combination with our VFH-hip and microarray data, suggest that Hh signals may act to promote the maturation or function of tolerogenic myeloid populations in the small intestine.

## Discussion

These data support the hypothesis that Hh signaling is an important epithelial modulator of inflammatory signaling in the small intestinal lamina propria. The transcriptional response of isolated E18.5 mesenchyme to both Shh and Ihh revealed immune response genes and inflammatory pathways among the top downregulated processes. Moreover, exogenous Hh ligand alone can reduce the strongly pro-inflammatory response observed during culture of isolated mesenchyme, indicating that epithelial Hh plays a major role in modifying inflammatory pathways. In accordance with this hypothesis, analysis of adult VFH-hip animals demonstrates that chronic reduction of Hh signals in the adult intestine leads to villus loss, spontaneous inflammation, and death. Taken together, these data provide the first direct evidence implicating epithelial Hh signals in modulation of inflammation in the small intestine, and provide insight into the role of this developmental signaling pathway in immune homeostasis in the gut.

One important trend emerging from the microarray experiments is the observation that Hh signals stimulate the expression of genes involved in the development and function of smooth muscle cells, including *MyoD*, *Igf1*, and *Fgf2*. This is an important observation in concert with extensive data indicating that Hh signals are crucial in smooth muscle development in the gastrointestinal tract,<sup>8–10</sup> lung,<sup>20</sup> bladder,<sup>21</sup> ureter,<sup>22</sup> and vasculature.<sup>23</sup> In addition, recent data from our group indicate that Hh

**Figure 4.** Hedgehog (Hh) ligand reverses up-regulation of pro-inflammatory genes that occurs in isolated mesenchyme. (A) K mean clustering analysis of gene expression from freshly isolated mesenchyme (Mes), cultured mesenchyme (Veh), and Hh ligand-treated cultured mesenchyme (Shh). Gene expression level is indicated by color, with blue indicating low expression, black moderate expression, and yellow high expression. Two clusters of genes that were low in freshly isolated mesenchyme, more highly expressed in cultured mesenchyme, and reduced with Hh ligand addition were visualized. (B) Genes that are both up-regulated by culture and down-regulated by Hh are overwhelmingly associated with inflammation and immune response.

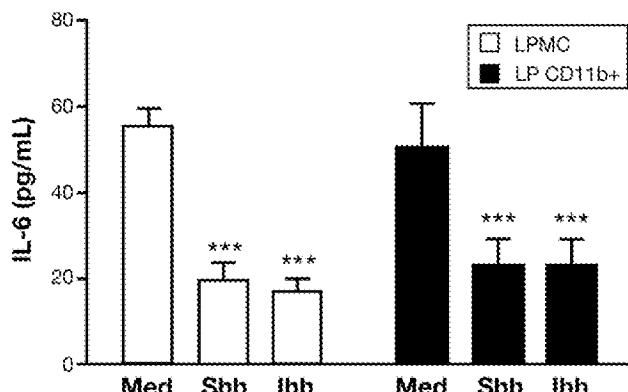


**Figure 5.** Small intestine myeloid lineages respond directly to Hedgehog (Hh) signaling. Co-staining in *Gli1*<sup>+/LacZ</sup> animals for nuclear LacZ and CD3 (A), CD11b (B), CD11c (C), and CD19 (D). Nuclei detected with anti-LacZ antibody are shown in red, and immune lineages are in green. Images shown are representative 0.3 μm confocal slices taken at 600×. Some CD11b<sup>+</sup> and CD11c<sup>+</sup> myeloid cells have LacZ-positive nuclei (arrows), while CD3<sup>+</sup> and CD19<sup>+</sup> lymphoid cells do not. The section in (D) is from the edge of a lymphoid nodule. (E, F) Staining of isolated intestinal mesenchymal cells in culture with anti-LacZ (red) and anti-CD11b (green). Overlay of red, green, and blue (4',6-diamidino-2-phenylindole [DAPI]) channels (E) and analysis of red channel alone (F) shows that CD11b<sup>+</sup> cells with stellate morphology (arrows) exhibit robust staining for LacZ. A second population of CD11b<sup>+</sup> cells with spread morphology (dotted circle) has lower nuclear levels of Gli1. Additional Gli1-positive cells are visible, likely myofibroblasts.

signals stimulate development of villus and muscularis mucosa smooth muscle through direct activation of the SRF co-activator, myocardin (Zacharias et al, submitted manuscript). The fact that myocardin and several other smooth muscle-related genes were also identified in our microarray experiments emphasizes the importance of Hh signals in the specification of smooth muscle cells.

The other major trend of our microarray studies was the unexpected identification of Hh ligands as key regulators of immune pathways in the intestine. Given the role of Hh in fate determination in multiple systems, it is tempting to speculate that the expression changes that we interpret as modification of inflammatory signaling actually represent modulation of the fate or activity of myofibroblast or myeloid cell lineages. Modulation of myeloid cell fate or phenotype would explain why addition of Hh, typically a ligand that promotes gene activation, down-regulates so many pro-inflammatory genes. The major pathways identified in our expression data are clearly associated with myeloid cell innate immunity, and our data demonstrate that myeloid cells respond directly to Hh signaling in the small intestine.

Myeloid cells are key determinants of inflammation in the intestine,<sup>3</sup> exerting regulatory control and communicating with other cell types to maintain proper immune homeostasis. Differential populations of myeloid cells are pro- or anti-inflammatory and these phenotypes can be modulated during response to external signals.<sup>4</sup> One intriguing possibility is that Hh signals help to create or maintain a proper balance of tolerogenic versus pro-inflammatory myeloid populations in the small intestine; this possibility will need to be formally explored in future studies. Additionally, myofibroblasts have been shown to secrete inflammatory mediators<sup>24</sup> and are responsive to Hh signaling throughout life.<sup>7</sup> Reduction in Hh signaling in late gestation<sup>8</sup> or postnatally (Zacharias et al, submitted manuscript) leads to mislocalization of myofibroblasts. Such changes in localization or function stimulated by reduced Hh may provoke altered inflammatory signaling from this regulatory population. Together, the myeloid and myofibroblast populations provide the best candidates for the cellular targets that receive Hh signals intended to regulate immune response and inflammation.



**Figure 6.** Hedgehog signaling inhibits intestinal lamina propria leukocyte interleukin (IL)-6 production. Lamina propria mononuclear cells (LPMC) or sorted CD11b<sup>+</sup> cells of C57BL/6 mice were isolated from the small intestine as described in Supplementary Methods. Cells were maintained in culture for 3 hours, then treated with Sonic Hedgehog (Shh; 250 ng/mL), Indian Hedgehog (Ihh; 250 ng/mL), or medium for 48 hours. The level of interleukin (IL)-6 was measured by enzyme-linked immunosorbent assay. Both Hh ligands inhibited the production of IL-6 by these two cell populations. \*\*\*P < .001.

Recent studies in the colon suggest that reduction in Hh signal transduction predisposes to inflammation in both mouse and human.<sup>6</sup> Our results here emphasize the point that functional Hh signals are required to maintain a tolerogenic milieu in the context of the mammalian small intestine. The stochastic nature of the development of significant inflammatory disease in VFHhip suggests that an unknown stimulus is needed to begin the inflammatory process. This stimulus may be the aberrant response to normal trauma in the absence of functional Hh signaling, a barrier breach due to villus loss, or another trigger. Regardless of the precise etiology, Hh signaling is clearly required to protect from such an event, as single transgenic littermates of VFHhip animals survive without inflammatory disease.

Hh has now been implicated in both colonic<sup>6</sup> and small intestinal inflammatory regulation. This is intriguing in light of significant recent studies demonstrating that some human susceptibility loci are associated with celiac disease as well as ulcerative colitis (eg, polymorphism in the IL2/IL21 region of the genome).<sup>25</sup> Strikingly, the inflammatory phenotype seen in VFHhip animals shares phenotypic similarities with both human Crohn disease and celiac disease. The inflammation is patchy and can be transmural, characteristics similar to Crohn disease. VFHhip animals also exhibit villus atrophy, crypt hyperplasia, and profound inflammation, mirroring a celiac-like phenotype. Moreover, the wasting disease experienced by older VFHhip animals may be the result of malnutrition secondary to lost absorptive surface after villus loss and inflammation; reduced absorptive surface is a hallmark of celiac disease in humans. Additionally, the prevalence of dermatitis in VFHhip animals mirrors the high incidence of dermatitis her-

petiformis in human celiac disease patients.<sup>14</sup> While the dermatitis in VFHhip animals may be a result of malabsorption or malnutrition, some VFHhip skin lesions demonstrate IgA deposition, a key finding in celiac-related dermatitis in humans. Finally, loss of smooth muscle may be a first step in the development of inflammation in VFHhip animals. Likewise, in humans with celiac disease, smooth muscle populations are affected; anti-smooth muscle antibodies are often found in celiac patients and may help identify a subset of those patients who are particularly susceptible to advanced disease.<sup>26</sup>

Our analysis of small intestinal immune cells that respond to Hh signaling indicates that CD11b<sup>+</sup> cells respond in vivo and in vitro to Hh ligand. Sorted populations of CD11b cells are functionally capable of responding to Hh signals by down-regulation of IL-6 protein and messenger RNA. Interestingly, there appear to be at least 2 different morphological subsets of these cells that demonstrate different levels of Gli expression. The Gli1<sup>High</sup>-expressing cells have the morphology of dendritic cells. Thymic dendritic cells have also been shown to express Gli1 and respond functionally to Hh.<sup>27</sup> Interestingly, although early studies indicated that T cells are also Hh-responding cells,<sup>28–30</sup> ablation of Hh signaling by knockout of smoothened in DN4 T cells has no effect on the differentiation or expansion of CD4 or CD8 populations.<sup>30</sup> Our finding that small intestinal T cells do not express Gli1 are in accord with these latter functional studies.

Overall, the data presented here provide novel evidence that Hh signaling is an important anti-inflammatory signal in the small intestine. The inflammatory milieu in the small intestine is specialized, and many studies have shown that tolerogenic signals from both the stroma and epithelium are critical in modulating the tolerogenic response of the small intestine innate immune system.<sup>3,31,32</sup> The emerging role of the Hh signaling pathway as important modulator of inflammation identifies an additional cellular signaling molecule from the epithelium as an important factor in balancing the inflammatory response of the mesenchyme. Hh signaling may cooperate with other intestinal tolerogenic signals (eg, TSLP) to pattern a proper intestinal inflammatory response, but these microarray studies show that Hh alone can dramatically alter inflammatory signaling in isolated mesenchyme. Reduction in this homeostatic Hh influence may predispose to a disordered inflammatory response even in the presence of an otherwise normal immune system and may contribute to human gastrointestinal disease.

## Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at [www.gastrojournal.org](http://www.gastrojournal.org), and at doi: 10.1053/j.gastro.2010.02.057.

## References

- Cho JH. The genetics and immunopathogenesis of inflammatory bowel disease. *Nat Rev Immunol* 2008;8:458–466.
- Taylor BC, Zaph C, Troy AE, et al. TSLP regulates intestinal immunity and inflammation in mouse models of helminth infection and colitis. *J Exp Med* 2009;206:655–667.
- Rimoldi M, Chieppa M, Salucci V, et al. Intestinal immune homeostasis is regulated by the crosstalk between epithelial cells and dendritic cells. *Nat Immunol* 2005;6:507–514.
- Zeuthen LH, Fink LN, Frokiaer H. Epithelial cells prime the immune response to an array of gut-derived commensals towards a tolerogenic phenotype through distinct actions of thymic stromal lymphopoietin and transforming growth factor-beta. *Immunology* 2008;123:197–208.
- Sun C, Hall JA, Blank RB, et al. Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. *J Exp Med* 2007;204:1775–1785.
- Lees CW, Zacharias WJ, Tremelling M, et al. Analysis of germline GLI1 variation implicates hedgehog signalling in the regulation of intestinal inflammatory pathways. *PLoS Med* 2008;5:e239.
- Kolterud A, Grosse AS, Zacharias WJ, et al. Paracrine Hedgehog signaling in stomach and intestine: new roles for hedgehog in gastrointestinal patterning. *Gastroenterology* 2009;137:618–628.
- Madison BB, Braunstein K, Kuizon E, et al. Epithelial hedgehog signals pattern the intestinal crypt-villus axis. *Development* 2005;132:279–289.
- Sukegawa A, Narita T, Kameda T, et al. The concentric structure of the developing gut is regulated by Sonic Hedgehog derived from endodermal epithelium. *Development* 2000;127:1971–1980.
- Ramalho-Santos M, Melton DA, McMahon AP. Hedgehog signals regulate multiple aspects of gastrointestinal development. *Development* 2000;127:2763–2772.
- Bai CB, Auerbach W, Lee JS, et al. Gli2, but not Gli1, is required for initial Shh signaling and ectopic activation of the Shh pathway. *Development* 2002;129:4753–4761.
- Chuang PT, McMahon AP. Vertebrate Hedgehog signalling modulated by induction of a Hedgehog-binding protein. *Nature* 1999;397(6720):617–621.
- Madison BB, Dunbar L, Qiao XT, et al. Cis elements of the villin gene control expression in restricted domains of the vertical (crypt) and horizontal (duodenum, cecum) axes of the intestine. *J Biol Chem* 2002;277:33275–33283.
- Hernandez L, Green PH. Extraintestinal manifestations of celiac disease. *Curr Gastroenterol Rep* 2006;8:383–389.
- Li X, Madison BB, Zacharias W, et al. Deconvoluting the intestine: molecular evidence for a major role of the mesenchyme in the modulation of signaling cross talk. *Physiol Genomics* 2007;29:290–301.
- Agren M, Kogerman P, Kleman MI, et al. Expression of the PTCH1 tumor suppressor gene is regulated by alternative promoters and a single functional Gli-binding site. *Gene* 2004;330:101–114.
- Niess JH, Brand S, Gu X, et al. CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. *Science* 2005;307(5707):254–258.
- Mason KL, Huffnagle GB, Noverr MC, et al. Overview of gut immunology. *Adv Exp Med Biol* 2008;635:1–14.
- Park HL, Bai C, Platt KA, et al. Mouse Gli1 mutants are viable but have defects in SHH signaling in combination with a Gli2 mutation. *Development* 2000;127:1593–1605.
- Miller LD, Wert SE, Clark JC, et al. Role of Sonic Hedgehog in patterning of tracheal-bronchial cartilage and the peripheral lung. *Dev Dyn* 2004;231:57–71.
- Haraguchi R, Motoyama J, Sasaki H, et al. Molecular analysis of coordinated bladder and urogenital organ formation by Hedgehog signaling. *Development* 2007;134:525–533.
- Yu J, Carroll TJ, McMahon AP. Sonic Hedgehog regulates proliferation and differentiation of mesenchymal cells in the mouse metanephric kidney. *Development* 2002;129:5301–5312.
- Passman JN, Dong XR, Wu S, et al. A Sonic Hedgehog signaling domain in the arterial adventitia supports resident Sca1+ smooth muscle progenitor cells. *Proc Natl Acad Sci U S A* 2008;105:9349–9354.
- Saada JI, Pinchuk IV, Barrera CA, et al. Subepithelial myofibroblasts are novel nonprofessional APCs in the human colonic mucosa. *J Immunol* 2006;177:5968–5979.
- Glas J, Stallhofer J, Ripke S, et al. Novel genetic risk markers for ulcerative colitis in the IL2/IL21 region are in epistasis with IL23R and suggest a common genetic background for ulcerative colitis and celiac disease. *Am J Gastroenterol*. 2009. Available at: <http://www.ncbi.nlm.nih.gov.proxy.lib.umich.edu/pubmed/19455118>. Accessed May 24, 2009.
- Pedreira S, Sugai E, Moreno ML, et al. Significance of smooth muscle/anti-actin autoantibodies in celiac disease. *Acta Gastroenterol Latinoam* 2005;35:83–93.
- Varas A, Hernández-López C, Valencia J, et al. Survival and function of human thymic dendritic cells are dependent on autocrine Hedgehog signaling. *J Leukoc Biol* 2008;83:1476–1483.
- Lowrey JA, Stewart GA, Lindey S, et al. Sonic Hedgehog promotes cell cycle progression in activated peripheral CD4(+) T lymphocytes. *J Immunol* 2002;169:1869–1875.
- Stewart GA, Lowrey JA, Wakelin SJ, et al. Sonic Hedgehog signaling modulates activation of and cytokine production by human peripheral CD4+ T cells. *J Immunol* 2002;169:5451–5457.
- El Andaloussi A, Graves S, Meng F, et al. Hedgehog signaling controls thymocyte progenitor homeostasis and differentiation in the thymus. *Nat Immunol* 2006;7:418–426.
- Smythies LE, Sellers M, Clements RH, et al. Human intestinal macrophages display profound inflammatory anergy despite avid phagocytic and bacteriocidal activity. *J Clin Invest* 2005;115:66–75.
- Denning TL, Wang Y, Patel SR, et al. Lamina propria macrophages and dendritic cells differentially induce regulatory and interleukin 17-producing T cell responses. *Nat Immunol* 2007;8:1086–1094.

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### Conflicts of interest

The authors disclose no conflicts.

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## Supplementary Materials and Methods

### Tissue Processing and Immunostaining

For histology, intestinal tissue fixed in 4% paraformaldehyde (PFA) at 4°C overnight, followed by dehydration and infiltration with paraffin. Eight-micrometer sections were stained with H&E. For detection of the intestinal absorptive surface, alkaline phosphatase was visualized using the Vector Red Alkaline Phosphatase Kit (Vector Inc, Burlingame, CA) following the manufacturer's protocol. For immunofluorescence, tissue was dissected in cold phosphate-buffered saline (PBS) and fixed for 30 minutes in 4% PFA at 4°C. After a brief rinse in PBS, the tissue was equilibrated overnight in 30% sucrose/PBS at 4°C and then embedded in OCT and frozen on dry ice.

Immunohistochemistry was performed on 8–10 μm frozen sections as previously described.<sup>1</sup> Antibodies used were hamster anti-CD3 (1:500; AbD Serotec, Raleigh, NC), goat anti-CD11b (1:500; Abcam, Cambridge, MA), fluorescein isothiocyanate-conjugated anti-CD11c (1:250; Abcam), and rabbit anti-β-galactosidase (1:2000; a gift of James Douglas Engel, Department of Cell and Developmental Biology, University of Michigan, Ann Arbor, MI). All antibody staining was performed overnight at 4°C, followed by incubation Alexa Fluor secondary antibodies from Molecular Probes, 1:1000 for 1 hour. Nuclei were counterstained with 6-diamidino-2-phenylindole (DAPI), and slides were mounted in Prolong Gold Antifade Reagent (Invitrogen, Carlsbad, CA). Digital images were taken using an Olympus FV500 confocal microscope at 600× for 0.30-μm optical sectioning.

### Mesenchyme Cultures for Microarray and Immunofluorescence Analysis

Mesenchyme was isolated as detailed previously<sup>2</sup> from E18.5 C57BL/6 embryos, using Cell Recovery Solution (BD Pharmingen, Franklin Lakes, NJ) at 4°C with gentle shaking. The isolated mesenchymal fragments were minced and cultured on collagen-coated plates in Dulbecco's modified Eagle's medium, 10% fetal bovine serum, 10 mM Hepes. At 24 hours, the medium was replaced. At 48 hours, the medium was again replaced, but this time, recombinant Hh protein was also added: 2.5 μg/mL recombinant mouse Ihh or Shh N-terminal polypeptide (Shh-N; R&D Systems, Minneapolis, MN). Parallel samples of cultured mesenchyme received vehicle (5% trehalose, 12.5 mg/mL bovine serum albumin, in PBS). After 24 hours of exposure to Hh ligand or vehicle, mesenchymal samples were harvested for microarray. For immunofluorescence, mesenchyme was cultured as above on collagen-coated coverslips (BD Biosciences, San Jose, CA), fixed for 30 minutes in 4% PFA in PBS at 4°C, and staining was performed utilizing the antibodies at the concentrations described here.

### Isolation of Intestinal Myeloid Cells

Small intestinal myeloid cells were isolated as previously described<sup>3</sup> with slight modification. Briefly, mouse small intestine was removed immediately after sacrifice, cut open longitudinally, sectioned into 2-cm pieces, washed with PBS, and incubated in PBS containing 2 mM EDTA and 1mM dithiothreitol for 20 minutes at 37°C. After a second 20-minute incubation, fragments were filtered through 100-μm strainer and then digested further by mincing into 1-mm cubes and incubation in 1 mM dithiothreitol, Liberase Blendzymes 3 (0.28 unit/mL; Roche, Basel, Germany), and 3% fetal bovine serum in RPMI-1640 for 20 minutes at 37°C on a shaker. The digested tissue was passed through a 100-μm strainer then a 40-μm strainer. The filtrate was spun down at 1500 rpm for 10 minutes. The isolated lamina propria cells were enriched further for mononuclear cells (LPMC) on a Ficoll gradient (GE Healthcare, Piscataway, NJ). CD11b<sup>+</sup> cells were sorted using MACS CD11b Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's instruction. LPMC or sorted CD11b<sup>+</sup> cells were maintained in culture for 3 hours, then treated with Shh (250 ng/mL), Ihh (250 ng/mL), or medium for 48 hours. The level of IL-6 was measured by enzyme-linked immunosorbent assay.

### Microarray Analysis

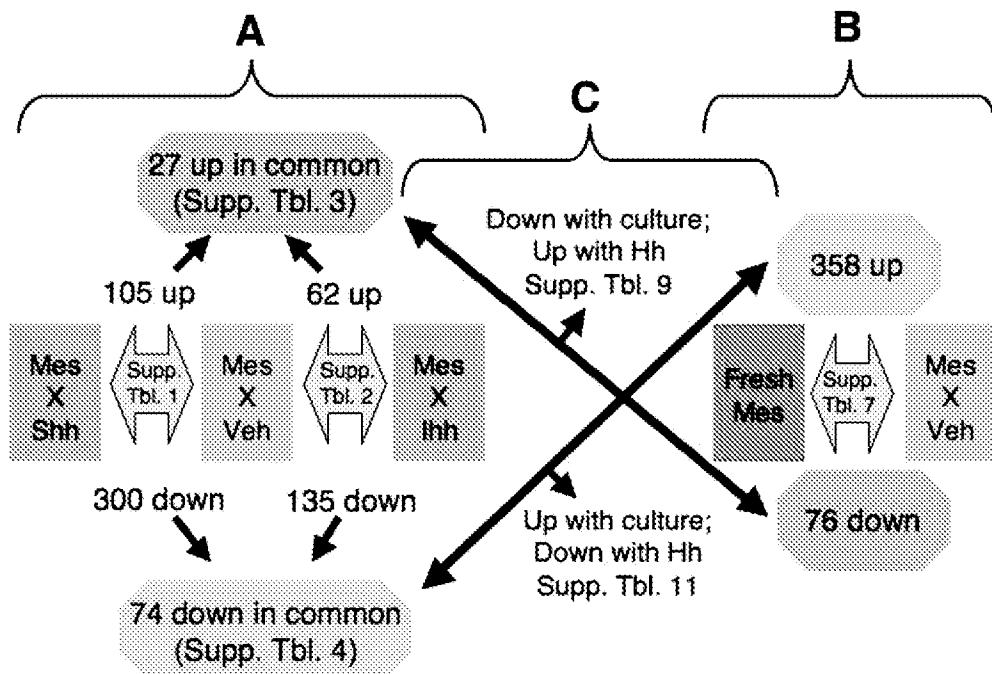
RNA was prepared from mesenchyme using Trizol (Invitrogen), following the manufacturer's instructions. Evaluation of RNA integrity, RNA labeling, and Chip hybridization was performed by the University of Michigan Affymetrix Microarray Core. RNA integrity was evaluated utilizing the Agilent 2100 Bioanalyzer system. Samples were hybridized to Affymetrix Mouse 430 microarray chips. After hybridization, expression values were normalized using the RMA and LIMMA functions of Bioconductor toolset in R. Fold changes were calculated using the LIMMA function, and *P* values were calculated using the Student's *t* test.

### Bioinformatics

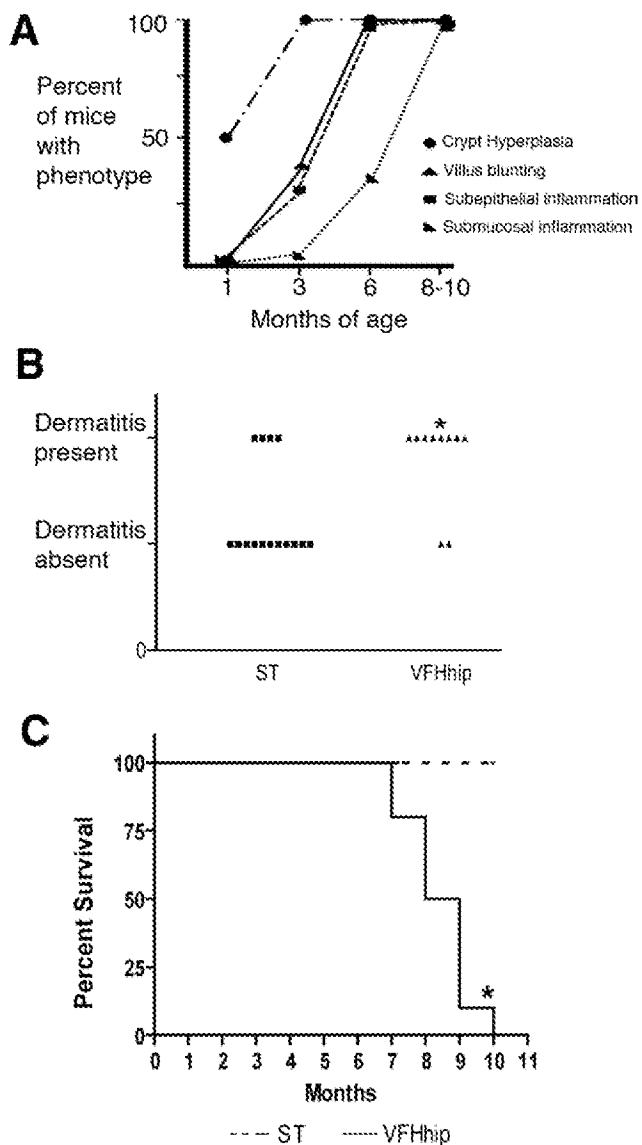
Comparisons performed are schematically diagrammed in Supplementary Figure 1. Examination of overrepresented Gene Ontology Terms and Functional Annotation Clustering analyses were performed using DAVID (<http://david.abcc.ncifcrf.gov/>) using described methods.<sup>4</sup> Categorization of overrepresented Gene Ontology Terms was obtained using the Gene Ontology Terms Classification Counter (<http://www.animalgenome.org/bioinfo/tools/countgo/>). MeSH filtration for identification of potential cellular enrichment was performed using the MeSH Anatomy filter in Bibliosphere ([www.genomatix.de](http://www.genomatix.de)). Hierarchical clustering analysis<sup>5</sup> was performed after RMA normalization using the K mean clustering function of MultiExperiment Viewer (<http://www.tm4.org/mev.html>).

## References

1. Kolterud A, Grosse AS, Zacharias WJ, et al. Paracrine Hedgehog signaling in stomach and intestine: new roles for hedgehog in gastrointestinal patterning. *Gastroenterology* 2009;137:618–628.
2. Madison BB, Braunstein K, Kuizon E, et al. Epithelial hedgehog signals pattern the intestinal crypt-villus axis. *Development* 2005;132:279–289.
3. Berndt BE, Zhang M, Chen G, et al. The role of dendritic cells in the development of acute dextran sulfate sodium colitis. *J Immunol* 2007;179:6255–6262.
4. Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protocols* 2008;4:44–57.
5. Eisen MB, Spellman PT, Brown PO, et al. Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci U S A* 1998;95:14863–14868.



**Supplementary Figure 1.** Schematic diagram of microarray comparisons. (A) Isolated mesenchyme was cultured for 48 hours to reduce the level of Hedgehog (Hh) signal transduction. Recombinant Sonic Hedgehog (Shh) or Indian Hedgehog (Ihh) was added with fresh medium and the cultures were incubated for another 24 hours. Cultures were harvested, and 3 independent samples each of Shh, Ihh, and vehicle-treated mesenchyme were used for microarray analysis. Probe sets altered ( $\geq 1.5$  fold,  $P \leq .05$ ) by Shh and Ihh are listed in Supplementary Tables 1 and 2, respectively. The number of probe sets that were up- or down-regulated by each treatment are listed in the figure. Probe sets up-regulated or down-regulated in common by both Shh and Ihh ( $\geq 1.5$  fold,  $P \leq .05$ ) are listed in Supplementary Tables 3 and 4, respectively. (B) A second microarray analysis compared freshly isolated mesenchyme to vehicle-treated, cultured mesenchyme. Probe sets robustly altered ( $\geq 5$  fold,  $P \leq .05$ ) are listed in Supplementary Table 7. In this analysis, 358 probe sets were up-regulated  $\geq 5$  fold by transition to culture, while 76 were down-regulated by this magnitude. (C) In a final analysis, we compared probe sets that were both down-regulated with transition to culture AND up-regulated by exposure to Hh ligand (Supplementary Table 9), as well as probe sets that were both up-regulated with transition to culture and down-regulated by exposure to Hh ligand (Supplementary Table 11).



**Supplementary Figure 2.** Clinical features of VFHhip mice. (A) Progression of disease in VFHhip mice. Individual disease features (crypt hyperproliferation, villus blunting, and inflammation in subepithelium and submucosa) were tracked at all ages in all mice. The graph plots the percent of mice with each symptom over time. (B) Presence of dermatitis in VFHhip mice (double transgenic) and signal transgenic (ST) littermates carrying either the Hhip or Cre transgene. VFHhip animals demonstrate dermatitis more frequently. (C) Lifespan analysis of VFHhip animals (solid line) and single transgenic littermates (dotted line). VFHhip mice show a significantly shortened lifespan compared to ST littermates.